

Euthanasia using gaseous agents in laboratory rodents

Ana Margarida Pereira#, Ana M. Valentim#, Silvana R. Guedes#, Luís M. Antunes

authors with equal level of contribution

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ABSTRACT

Several questions have been raised in recent years about euthanasia of laboratory rodents. Euthanasia using inhaled agents is considered an aesthetic method, and it is possible to be applied to a large number of animals at the same time. Nevertheless, its aversive potential has been criticized in terms of animal welfare. Available data regarding the use of CO₂, inhaled anaesthetics such as isoflurane, sevoflurane, halothane and enflurane as well as carbon monoxide and inert gases are discussed throughout this review. Euthanasia of foetus and neonates is also addressed. A table was assembled with available information to ease access to data regarding euthanasia techniques with gaseous agents in laboratory rodents. Regarding a better animal welfare, currently, there is insufficient evidence to advocate banning or replacing CO₂ in euthanasia of rodents, but there are hints that alternative gases are more humane. The previous exposure to a volatile anaesthesia gas before loss of consciousness is proposed by some scientific studies to minimize distress, however, the impact of such measure is not clear. Areas of inconsistency within the euthanasia literature have recently been highlighted and related to insufficient knowledge, especially on the advantages of the administration of isoflurane or sevoflurane over CO₂, or other methods, before loss of consciousness. Alternative may pass by the development of techniques to induce death in animals' home cage in order to minimize distress. Scientific outcomes have to be considered before the choice of the ideal method in order to obtain the best results and accomplish the 3R's.

Key words: euthanasia, laboratory rodents, welfare, carbon dioxide, volatile agents

INTRODUCTION

The word euthanasia is derived from the Greek term "eu" meaning good and "thanatos" meaning death. A "good death" would be one that occurs with minimal pain and distress. For animals, the term euthanasia is often substituted by terms such as humane death or humane killing. For simplicity, throughout this review the term euthanasia will be used.

INSTITUTO
DE INVESTIGAÇÃO
E INOVAÇÃO
EM SAÚDE
UNIVERSIDADE
DO PORTO

Rua Alfredo Allen, 208
4200-135 Porto
Portugal
+351 220 408 800
info@i3s.up.pt
www.i3s.up.pt

Laboratory rodents are euthanized for various reasons: to provide tissues for scientific purposes, at the end of an experiment, when adverse effects (pain, distress, suffering, etc.) become excessive, and when animals are unwanted stock (1).

Gas killing is one of the techniques used for rodent's euthanasia. It has advantages for the operator and for the animals. However, the onset of LOC (loss of consciousness) may be delayed comparing with other techniques and the question arises to what extent the exposition to gas induces distress or even pain. Carbon dioxide (CO₂) is widely used for euthanasia of rodents; however, concerns have emerged that CO₂ may induce pain or distress. The ongoing controversial discussion suffers from the lack of updated reviews of the effects of CO₂. Inert gases and inhaled anaesthetics have been pointed as a better option to induce unconsciousness prior to the administration of CO₂, although time to death is largely delayed using volatile gas anaesthetics. Coenen suggestion of minimize pain and distress rather than enhance a fast LOC (2), is being addressed and supported by many researchers. This review summarizes published works in which gases are used as euthanasia agents for rodents. A table was compiled with available information to ease access to data regarding euthanasia methods with gaseous agents in laboratory rodents.

Euthanasia Methods

It is interesting to note that ideal euthanasia techniques using chemical agents have much in common with the best practice in anaesthesia. Good anaesthesia practice is based on the simultaneous existence of three reversible components: unconsciousness, analgesia and muscle relaxation. The best euthanasia techniques aim for the induction of rapid unconsciousness followed by fast death, which can be effectively achieved by a physical method. Therefore, the period before LOC is a main concern in euthanasia since, animals may experience distress, anxiety, apprehension and pain. These latter factors may be reduced by proper handling of animals before euthanasia. The use of home cages, consistent group compositions (cage-mates rather than strange animals) and performing euthanasia in a room with no signs/odours of blood are positive examples of such handling conditions. The considerations of operator safety and aesthetics of method are also a concern. There is a risk of the operator feel an emotional burden and refuse to perform the euthanasia, not because it is inhumane for the animal, but because it is unaesthetic.

The most common techniques accepted for adult rodents are divided into chemical, and physical methods. The latter should provoke an immediate loss of consciousness due to the impact on the brain, and may cause less distress to the animal. However, it requires animal handling and restrain, which induce distress (3). Cervical dislocation, cerebral concussion, decapitation and microwave irradiation using appropriate equipment are accepted methods under certain conditions (4). Physical methods have the disadvantage of requiring training, which increases the possibility of errors during the killing process, and thus may fail the achievement of rapid LOC. Physical methods are considered a time-consuming and not aesthetic technique (5). If well performed, it may provide a fast and likely humane death, however, their use is limited for euthanasia of a large number of animals.

Chemical methods include inhalational or injectable agents. Barbiturates and sodium pentobarbitone are the most commonly used and accepted agents for euthanasia. Injectable anaesthetic agents may be used for euthanasia when employed at doses far higher than those used for anaesthesia, leading to overdose (6). The administration route, intraperitoneal, intravenous or subcutaneous, should be considered when selecting the doses. The administration itself is a source

of distress, since it involves withdrawing the animal from the home cage, handling and restrain it to perform the injection (7). Injectable anaesthetic agents are an aesthetical method, but its use is limited for mass killing, and has the disadvantage of requiring expertise, careful handling and proper restrain.

Additional information may be found in reports from several working groups in which recommendations for euthanasia of laboratory animals in Europe (1, 8) and in the USA (4, 9) are provided.

Inhalational gases such as halogenated anaesthetics, inert gases (argon and nitrogen) and CO₂ have been suggested as euthanasia agents. Its use requires placing the rodents inside a gas chamber to be filled with the inhalational substance. The volume and concentration of gases administered are controlled by a flow meter and a calibrated vaporizer. Waste anaesthetic gas should be scavenged to protect the operator (8). The use of inhalational agents requires equipment, which may be a disadvantage; however, it enables mass killing with good results regarding animal welfare. Furthermore, it requires minimal animal handling compared with physical and other chemical methods already described. The importance of inhalational agents in euthanasia refinement is further discussed below.

Carbon dioxide

Carbon dioxide has been used to euthanize groups of rodents in specially designed chambers. Among the advantages stressed for the continuing use of CO₂ alone are the fact that it is a practical and effective technique with a good cost-benefit compromise. Evidence from human studies though has shown that inhalation of CO₂ at different concentrations cause pain and/or distress (10). According to Leach, in humans, rats, and cats, most nociceptors are activated at a concentration of approximately 40% of CO₂ (11). Moreover, in mice, CO₂ at a concentration of 10% has been shown to evoke fear behaviour by expressing freezing and activation of the limbic structures, including the amygdala (12). Inhalation of CO₂ causes respiratory acidosis and produces a reversible anaesthetic state by decreasing the intracellular pH (4). Physiological effects/actions of CO₂ are revised elsewhere (13). To clarify CO₂ suitability for rodent euthanasia, several studies have been performed in which the use of CO₂ is addressed. During CO₂ exposure, time to achieve unconsciousness is dependent on the concentration, chamber volume, and flow rate at which the gas is delivered. In rats, unconsciousness is achieved at CO₂ concentrations of 30-40% (14) however no data is available for mice. Other studies in mammals and birds described that LOC is achieved at higher CO₂ concentrations (>40%), while for killing it should be above 70% (11). Even though euthanasia using 100% CO₂ pre-filled chambers induces a rapid loss of cortical brain activity within 39 seconds in rats (15) and 30 seconds in mice (16), it is consider unacceptable (1) due to the significant pain inflicted until LOC. A recommended and accepted procedure is to place the animals into a chamber containing room air followed by a gradual-fill of CO₂ (9). In this sense, the determination of the gas flow rate is critical to enhance a humane use of CO₂ (4, 16, 17). Table 1 shows available information regarding euthanasia studies performed with several flow rates.

It is common to assess subjective experiences of animals to answer scientific questions (18). The assessment of animal welfare is usually done by studying animals' emotions, analysing their behaviour and decisions when facing a certain environment. Aversion is a negative emotional response, described by humans, for example, when experiencing dyspnea (19, 20). Several studies to assess gas aversion used approach-avoidance paradigms, consisting on providing a goal that is both appealing (presence of rewards) and unappealing (presence of gas). Thus the animal has to make a compromise between the two stimulus, depending on the aversion or motivation degree of each one. Results from approach avoidance test indicate that the latency to leave the CO₂ chamber was lower compared with the time to LOC with flow rates ranging from 3% to 27% V min⁻¹ (21-23). Some studies showed that rats receiving a flow rate of CO₂ 17% V min⁻¹ expressed signs of avoidance after one minute of exposure (24), while a gradual displacement of 14% (23) and 10% V min⁻¹ (25) appears to be less aversive. In addition, it was described in rats that, by the administration of CO₂ at a flow rate of 17.25% V min⁻¹, recumbence was achieved after 106 seconds. At this moment, the concentration of CO₂ inside the chamber was approximately 33%, which is under the pain threshold for the majority of the nociceptors located in the nasal mucosa. It suggests that animals did not feel pain related to the procedure, although there were signs of distress like increases in the frequency of rearing, escape behaviour, vocalizations and in the time spent with the nose contacting the chamber lid (26). Makowska reported that rats escaped from an environment containing CO₂, when its concentration reached 13.5-18.2% (22). Another study from Niel showed similar results, rats left the gas chamber when CO₂ concentrations reached on average 18.4% (24). These studies suggest that, even during gradual-fill procedures with low concentrations, aversion arising from mechanisms other than pain may cause distress. Animals placed inside a chamber with rising concentrations of CO₂ may find it aversive and may experience dyspnoea and "air hunger", which is known to be very distressing in humans (27). Some other adverse effects documented with CO₂ exposure are gasping/forced breathing pattern in rats (14) and increased in dyspnoea scores in mice (28). In contrast to the use of low flow rates, a recent study advocate that a flow rate of 50% V min⁻¹ with a concentration of CO₂ inside the chamber below 40% reduce dyspnoea onset and insensibility and therefore, stressful events (29).

The addition of nitrous oxide (N₂O) or oxygen (O₂) was proposed to improve CO₂ exposure, trying to reduce the onset of unconsciousness and dyspnoea, respectively. Nitrous oxide worked as a carrying gas for CO₂; this second-gas effect shortened the time to LOC in 10% compared to the use of CO₂ alone (30). Oxygen was added to prevent hypoxia, thus reducing distress. At high concentrations (30%), O₂ causes hyperoxia, which reduces the ventilator and dyspnoea responses to hypercapnia (2, 31, 32). However, studies showed that the addition of O₂ to CO₂ causes only a slight reduction of CO₂ aversion in the gradual-fill procedure (33) or this procedure resulted in the same degree of aversion (34). Moreover, it was shown that O₂ supplementation may provoke lung haemorrhage before LOC in mice (35).

Even though, consensus towards the use of CO₂ has not yet been reached, there are recommendations based on data from the presented studies. It is recommended the use of compressed CO₂ in cylinders combined with calibrated flowmeters, which allow the precise regulation of the inflow to the chamber. The gas flow should be constant at rate of 15 to 35% V min⁻¹, and it may be increased after LOC in order to speed up death. Users should wait a minimum of 49 seconds before increasing the flow rate of CO₂ (36). The gas flow should be maintained for at least one minute after apparent clinical death (4).

Volatile anaesthetics

The use of inhaled anaesthetics to induce unconsciousness has been pointed as a more humane technique for euthanasia than CO₂ as

these agents might be less aversive and do not inflict pain. Nonetheless, there is no consensus on whether distress during induction is less with these agents than with CO₂. In addition, scavenge systems for the elimination of waste gas is mandatory to protect personnel from exposure to the anaesthetic gases which have health and safety implications. Air or O₂ should be provided during induction when using volatile anaesthetic agents to avoid hypoxia (6). As agents have to reach a certain alveolar concentration before they become effective, this method takes some time, during which animals may suffer distress. Animals may struggle and become anxious during induction of anaesthesia because drugs may be irritant and can cause excitement between the beginning of the procedure and LOC (4). The expression of distress due to anaesthetic properties (odour, hypoxia, hypercarbia) may be hard to isolate from the expected excitatory phase of anaesthesia induction, when animals increase their activity and speed of movements. Excitation is also observed when injectable anaesthesia is performed, but not jumping, making this a behaviour more related with distress induced by volatile agents (30).

Isoflurane is a commonly used anaesthetic in most laboratories, less soluble than halothane, and it should induce anaesthesia more rapidly. However, it has a slightly pungent odour (37) and animals often hold their breath, delaying the onset of LOC and increasing levels of distress (4). As halothane has a lower MAC and higher potency compared with isoflurane, a greater quantity of isoflurane may be required to kill an animal (4, 38). Although isoflurane is acceptable as a euthanasia agent, halothane is less irritant and the odour is not so intense causing less disturbance in the respiratory airways, at least in humans (38). A great disadvantage of halothane is the difficulty to find it in the market nowadays.

Leach and colleagues showed that the level of aversion was proportional to the increase of isoflurane and halothane concentrations (39) and recommended the use of a medium concentration of halothane for rats and halothane or enflurane for mice (34, 39). A study of Makowska and colleagues using approach-avoidance testing showed that most rats reach ataxia, a state of conscious sedation, before choosing to leave a cage gradually filled with isoflurane or halothane, without, however, find differences between both anaesthetics (40). Another study of the same group compared several inhaled agents and showed that mice took more time to leave atmospheres containing isoflurane than halothane (22). Moreover, two mice remained in the isoflurane chamber until recumbency, suggesting that isoflurane may be an alternative to CO₂ (22). The different outcomes of Leach studies compared with Makowska studies may be explained by the use of pre-filled chambers, inducing an unpleasant contact with high concentrations of isoflurane, known for its pungent odour

compared with halothane. Moreover, Leach did not use rewards, contrary to the studies of Makowska, which could have influenced the latency to leave the gas chamber measured in these studies. Thus, in the presence of food rewards, two animals achieved LOC in the isoflurane chamber, because the potential aversion induced by isoflurane was lower compared with the degree of motivation to eat the reward.

Probably due to different approaches and interpretations there are, in fact, some contradictory literature regarding isoflurane. Valentine reported that the use of isoflurane induction prior to CO₂ euthanasia considerably increased c-fos expression in the brain, which has been described as a neural marker of pain and related with distress. Agitation scores were also found higher with isoflurane than with a recommended flow rate of CO₂, (20 Vmin⁻¹), without, however, alterations on plasma ACTH and corticosterone (28). Different outcomes were obtained by a study comparing the effect of isoflurane and a CO₂:O₂ mixture on corticosterone in rats during serial blood collections. This indicated that, after one hour, a significantly lower corticosterone concentration was observed when isoflurane anaesthesia was used compared with CO₂ (41). Lower concentrations of corticosterone suggests that animals had experienced less distress prior to LOC. Although these different results, several authors agree that isoflurane represents a refinement over the exposure to CO₂ alone for euthanasia (36, 42, 43). However, it only applies if no previous exposure to the anaesthetic had occurred, as re-exposition to isoflurane and sevoflurane induced aversion behaviour in rats measured by a decrease number of animals that stayed or took longer to leave the gas compartment (42, 44).

Sevoflurane is less soluble than halothane and does not have an objectionable odour, but it is less potent than isoflurane or halothane and has a lower vapour pressure. Recent studies from our group (45) suggest that mice have a low degree of aversion to sevoflurane, as they spent more time in the sevoflurane chamber, where food rewards were presented, than in the chamber filled with environmental air; this did not occur to CO₂ or isoflurane. Actually, mice showed to spend less time in the isoflurane chamber with food rewards than in the chamber with air, indicating aversion to this gas. Contrary to this, a recent study advocate that rats find sevoflurane and isoflurane similarly aversive (44), probably due to the use of higher concentrations than the ones used in our study (45). Studies with different concentrations and flow rates may be required to understand smaller differences between these two halogenated. Although the presented information, the volatile anaesthetic gases still induce some degree of aversion in rodents. It would be of great interest to get more information about the advantages of the use of more recent inhaled anaesthetics such as sevoflurane, desflurane or enflurane, completing and consolidating the information already presented by Leach and colleagues (39).

Inert gases

Nitrogen (N₂) and argon (Ar) are inert colourless and odourless gases, with no flammable or explosive properties. For euthanasia, a container is usually pre-filled with a minimum of 98% by volume of N₂ or Ar to induce death by hypoxemia. As N₂ is lighter than air, specialised equipment is needed for its administration; Ar is denser than air, and easily contained. Studies by Leach found that rodents showed less aversion to Ar compared with CO₂, which may be due to its odourless, tasteless and inert properties. However, animals could enter and leave the chamber at will, and so LOC was never achieved (34, 39).

Early approach-avoidance tests with food rewards show that rats are able to detect decreases in O₂ concentration almost immediately after Ar delivery began, and they stop eating when hypoxia become sufficiently aversive (46). In another study, rats refused to enter in a chamber containing Ar and none of them ate food rewards, which highlight the aversive properties of Ar (24). It is possible that cognitive impairments, dizziness and visual changes associated with low O₂, are the cause of rats' aversion to Ar (46). Physiological effects of hypoxia become aversive at approximately 7.7% O₂; however, these O₂ concentrations are too high to cause unconsciousness or death (46). Thus, according with these findings, an effective oxygen concentration to argon euthanasia would always be aversive. Indeed, studies showed that argon induced back arching with an open mouth in rats, i.e. abnormal gasping (25). Rats exposed to Ar and N₂ exhibited muscle spasms and were hyperreflexic to touch and handling when they appeared unconscious. Prolonged tachycardia following short-term exposure are also associated with Ar (47). Similarly, N₂ at approximately 100% was not very effective, as it was slow to produce unconsciousness and death and also induced hyperreflexia during short-term exposure (47).

There is still no consensus in the guidelines and legislation regarding the use of Ar or N₂. In the American Veterinary Medical Association Guidelines on euthanasia, these techniques are conditionally acceptable, and so only used if O₂ concentrations <2% are achieved rapidly, and animals are heavily sedated or anesthetized (4). On the other hand, this method is accepted in the European Directive (49). Either way, it has to be considered the welfare implications to use inert gases regarding asphyxiation, which can cause alveolar haemorrhage, and the displacement of oxygen inducing hypoxemia before loss of consciousness.

Carbon monoxide

CO binds irreversibly with haemoglobin to form carboxyhaemoglobin and blocks uptake of O₂ by erythrocytes, finally leading to unconsciousness and fatal hypoxemia (4). Rodents should be placed inside a container pre-filled with at least 6% CO by volume. CO is highly explosive above 10% and toxic to operators; hence it must only be used with appropriate gas scavenging. Commercially compressed CO is preferable to CO generated by other means, because it is not contaminated with other gases, and it minimizes problems associated with adjusting the concentration, cooling of the gas and equipment maintenance. In addition, personnel must be instructed thoroughly the use of CO, in order to understand its hazards and limitations (4).

Concerning aversion in rats, a study showed that intermediate and high flows of CO provoked the recumbence of two animals in a situation where they could escape to another cage. However, these rats exhibited convulsions, and it was not clear if they were completely unconscious when this occurred. The other animals showed behaviour changes such as agitation, which suggests aversion to CO exposition (48). Therefore, there are no clear evidences that CO can be used as a refinement in euthanasia. Furthermore, it can be dangerous for the operator.

Euthanasia of the Fetuses and Newborn Animal

There is not much new data regarding recommendation for foetuses and newborn animals euthanasia. Nevertheless, there are two factors that must be taken into account when choosing a euthanasia method for the fetal or newborn animal: they are resistant to hypoxia, and they metabolize drugs slowly.

The specificity of euthanasia recommendations for foetuses is based on its neuronal development. Foetuses up to 15 days are believed to have minimal pain perception due to a non-functional cerebral cortex and subcortical brain structures (4, 50). Thus, to kill the mother is enough to cause a rapid death of the foetuses as they are non-viable at this stage of development (51). In rats and mice over 15 days after conception, pain is perceived so humane methods of euthanasia should be chosen. Skilful injection of chemical anaesthetics, decapitation with surgical scissors or cervical dislocation are accepted; inhalant anaesthetics or CO₂ can be used, however, it requires long time of exposure and distress induction is a risk (51). When foetuses are not used for further experiments, mother euthanasia should ensure cerebral anoxia and minimally uterine disruption, for example by using CO₂ euthanasia followed by cervical dislocation (51).

In the case of neonatal rodents, recent evidence confirms that there is a huge difference in the time until death with CO₂ compared with adults (52) due to the resistance of neonates to hypoxia. In rats, the time to death decreased steadily with increasing age, with 100% of the rats euthanized after 5 min of CO₂ exposure at 10 days of age. The time required for 100% of mortality decreases by 3 min for each day of age between days 0 and 10 (53). The methodology for euthanasia of neonatal animals with CO₂ must therefore be substantially modified from that employed for adults. The euthanasia techniques acceptable in neonates are injection of chemical anaesthetics (e.g. pentobarbital), cervical dislocation, or decapitation (54). In these rodents, decapitation can be performed with a sharp knife or scissors. The bilateral pneumothorax method may be used as a secondary method to ensure death in anaesthetized newborn. Immersion in liquid nitrogen is used only if preceded by anaesthesia (4, 6), but it is considered acceptable if foetuses or neonates do not have fur and have less than 4 grams (1). The guidelines/ acceptance of this technique may be different between countries: for e.g. in Switzerland, rapid freezing is allowed without anaesthesia in fetal and newborn animals below 10g of body weight (55). The most suitable euthanasia method for rodents may also differ depending on the strain (52).

Equipment

The most common euthanasia solutions involve the use of anaesthesia equipment which includes the anaesthetic chamber, vaporizers, scavenging system and obviously, gases. Although the anaesthesia equipment available in the laboratory may be practical to euthanize few animals, it may not be the most adequate one for mass killing in a daily basis. The choice of equipment for euthanasia of rodents should take into account both animal welfare and personnel safety, which is achieved by minimizing the human occupational exposure to the agents. Although there are different recommendations among countries, the concentrations of halothane, enflurane, and isoflurane to which humans are exposed should be less than 2 ppm, and 25 ppm for N₂O (56, 57). Hence, it is of the most importance to perform the procedure using properly designed equipment with a well-designed waste gas scavenging system to collect, remove, and dispose the gases.

From a welfare point of view, the equipment should reproduce a correct adjustment of the flows, according to the recommendations for each gas and be as silent as possible, since noise, and the stream of inflowing gas may induce distress. However, it is known that introduction of the animals into a gas chamber causes physiological and behavioural changes in rats (25), which are evidence of distress. Therefore, it raises an interest on developing solutions that avoid the need to handle or move the animals. In response to this concern, new products were engineered in order to permit to euthanize the animals in their home cages, by developing mobile or fixed euthanasia stations and also automated devices connected to lids that are adapted to the commonly used makrolon cages (58). This equipment runs different cycles, using CO₂ as single euthanasia agent or in combinations with previous administration of isoflurane.

In the future, systems with automatic recognition of LOC may be available to be used during the induction phase of euthanasia (59). After LOC is recognised, euthanasia may be concluded with a potential more aversive gas or with a rapid increase concentration of the first gas used able to kill the animal quickly.

Another important feature is to enable a cost-effective euthanasia with a sized-adjusted equipment to the number of animals to euthanize which minimizes waste of anaesthetics and therefore, costs.

Conclusions

Areas of inconsistency within the euthanasia literature have recently been highlighted and related to insufficient knowledge of the best methods of euthanasia for various species and strains at different life stages. For practical reasons, and often also for research considerations, depending on the species and number of animals, LOC is achieved with an anaesthetic, and then rodents may be killed by switching the agent to CO₂, or by using an injectable agent or physical method afterwards (14).

A great deal of research remains to be done on the topic of laboratory animal euthanasia and rodents in particular. The information available is based mainly on rat studies, and more new studies using mice are needed to avoid the extrapolation of information between species. Furthermore, there is a lack of consensus between individual opinions regarding the best euthanasia techniques, which may reflect the wide range of experience of users of these techniques as well as high variability related with subjective concepts (e.g. distress, pain, level of expertise). Many improvements to current methods could be made, including use of home-cages euthanasia (58), and the implementation of gas chambers with fill rates or gas mixtures tailored to minimize distress. Alternative gaseous agents need further evaluation. However, there is a theory that, at least rats, avoid anything that produces a state change (60). Hence, even if the euthanasia agents are not aversive per se, the novel state of conscious sedation may induce fear (22). In agreement, all the different gases may never be perfect and always have a disadvantage associated. Administration of isoflurane or sevoflurane prior to CO₂ is pointed as a more humane death, but there is still no consensus and the information on the advantages of sevoflurane is little. However, a move away from CO₂ faces two obstacles: practicality and economics (61) as anaesthesia-based techniques require more time, drugs and equipment. Inert gases do not seem to be less aversive than CO₂. However, the combination of different gases that potentiate LOC in short time, as N₂O and CO₂ (30), has been pointed as an euthanasia refinement, and other combinations as CO₂ and volatile gases would be of interest to be further studied.

In conclusion, there is no evidence to advocate banning CO₂, though its flow rate should be low. However, evidences have been pointing to a potential refinement using volatile gas anaesthetics. The use of these agents increases euthanasia costs. Therefore, the use of a bi-phased euthanasia, in which LOC achieved with these anaesthetics is the first objective, and death achieved with CO₂ the second, has been shown to be advantageous regarding better animal welfare, practicability and cost. Furthermore, different anaesthetics need to be further evaluated, such as sevoflurane and desflurane. Beside the gaseous agents choice, euthanasia refinement may be also achieved by the development of techniques to induce death in animals' home cage in order to minimize handling and distress.

Declaration of conflicting interests

None to declare.

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Table 1: Summary of euthanasia studies in mice and rats with several agents and flow rates.



Gas	Ref	Specie & strain	[Concentration]/Flow rate (V min ⁻¹)	[Estimated Concentration] / Time to LOC (s)	[Estimated Concentration]/ Time to death (s)	Parameters measured	Conclusions	Comments/ Recommendations
CO ₂	Niel, 2008 ²⁴	♂ Wistar rats	GF 17%	NA	NA	Approach-avoidance (food rewards).	Rats showed avoidance and escape responses to CO ₂	Re-exposure to CO ₂ does not cause habituation.
	Niel, 2008 ²⁵	♂ Wistar rats	GF 3%; 7%; 14%; 27%	NA	NA	Approach-avoidance (food rewards).	A flow rate of 14% V min ⁻¹ is optimal in terms of initial aversion; after this initial aversion (forced exposure) it induces distress with all flow rates.	
	Moody, 2014 ¹⁸	♀ albino C57BL/6J-Tyr mice	GF 20% *	119.2 ± 10	NA	Dyspnoea onset; LOC; LOPWR.	Gradual-fill with higher flow rates reduce period from onset of dyspnoea until LOC; thus it is a refinement.	When using higher flow rates, a gas holding technique should be used to ensure that painful CO ₂ concentrations [>40%] are
			GF 30% *	98.8 ± 11				
			GF 40% *	110.3 ± 10				

Table 1: Summary of euthanasia studies in mice and rats with several agents and flow rates.



			GF 50% *	106.2 ± 10				not reached until insensibility occurs.
	*After LOPWR, a flow rate of 60% V min ⁻¹ was delivered to speed up death.							
CO ₂ Iso	Wong, 2013 ⁴²	♂ Sprague–Dawley rats	24% CO ₂ [5%] Iso 100% O ₂	NA	NA	Aversion behaviour (dark/light compartments).	Iso is a refinement over CO ₂ exposure; though its re-exposure is as aversive as CO ₂ .	
	Valentine, 2012 ¹⁷	♀ CD1 mice	20% CO ₂ 100% CO ₂ [5%] Iso in 20% O ₂	NA	NA	<i>c-fos</i> in the brain; ACTH; corticosterone; behaviour.	20% V min ⁻¹ CO ₂ alone is the most humane method of euthanasia for mice.	
			GF CO ₂ 20%	NA	NA			

Table 1: Summary of euthanasia studies in mice and rats with several agents and flow rates.



	Moody, 2014 ⁴³	♂ C57BL/6J mice	Iso [5%] in 40% O ₂			Aversion behaviour (dark/light compartment s).	Isoflurane is an alternative to CO ₂ exposure; however it should be avoided if recent exposure occurred.	It is suggested to use [5%] Iso delivered at a rate of 40% V min ⁻¹ to induce LOC.
	Moody, 2015 ³⁶	♂ C57BL/6J mice	20% CO ₂ *	15.7 ± 10.9	114.8 ± 5.8	Time to recumbency; Time to LOC; time to LOPWR.	Isoflurane is a humane alternative to CO ₂ exposure.	Time to LOPWR was included in this table as LOC measure.
			[5%] Iso in 17% O ₂ *	40.4 ± 12.9	222.1 ± 5.4			It is recommended to wait a minimum of 79 s after the appearance of recumbence before switching to a high flow rate of CO ₂ .
	*After LOC a flow rate of 60% V min ⁻¹ was delivered to speed up death.							
CO ₂ Iso Hal o	Makwoska, 2009 ²⁶	♂ CD-1 mice	GF 18% - 70% CO ₂	NA	NA	Approach-avoidance (food rewards).		Each group involved in the preliminary testing of anaesthetics were re-exposed after 20h.
			GF 66% - 160% Ar	NA				All animals exposed to CO exhibited convulsions after they were in recumbency,
			GF CO [8%] in air	[5.1 ± 0.4%] /				

Table 1: Summary of euthanasia studies in mice and rats with several agents and flow rates.



Ar CO				39 ± 5				but it is not clear if they were unconscious when this occurred.
			GF [3%], [4.5%] Halo in 70% O ₂	93 ± 9 Re-exposition: 68 ± 5				
			GF [2%] [3%] Iso in 70% O ₂	100 ± 4 Re-exposition: 64 ± 8				
CO ₂ Ar	Niel, 2006 ²⁹	♂ Sprague Dawley rats	GF 17.25% CO ₂	[33%] / 106 ± 12	[80%] / 443 ± 14	Behaviour (activity, rearing, nose to lid, escape behaviours, vocalization).	GF CO ₂ euthanasia causes distress in rats, and the concentrations involved suggest that this distress is due to dyspnoea rather than pain. Nose to lid slightly increased when exposed to Ar.	[CO ₂] tended to be greater at the bottom [9.4%] of the chamber than at the top [2%].
			GF 17.25% Ar	> 105	NA			
	Niel, 2007 ²⁷	♂ Wistar rats	PF [5%] CO ₂	NA	NA	Approach-avoidance	PF and GF CO ₂ exposure, and 90% argon exposure cause aversion in rats.	Rats tolerated extended exposure to 5% and 10% CO ₂ , but this was not
			PF [10%] CO ₂					

Table 1: Summary of euthanasia studies in mice and rats with several agents and flow rates.



			PF [15%] CO ₂			(food rewards).		sufficient to cause unconsciousness.
			PF [20%] CO ₂					
			GF CO ₂ 17%					
			PF [90%] Ar in Air					
	Burkholder, 2010 ²⁸	♂ Sprague Dawley rats	GF 10% CO ₂	[21% ± 2%] 156 ± 12	NA	Physiological parameters (temperature, heart rate, and activity); behaviour; Pathologic examinations (lungs, nares, brain, adrenals).	Ar and CO ₂ induce stress; however CO ₂ is preferable to Ar.	
			GF 50% Ar	[100%] 138 ± 41				
CO ₂	Thomas, 2012 ³¹		GF 20% CO ₂	[24%] CO ₂ 108.76 ± 9.4	NA	Time to LOC; blood analysis	The addition of N ₂ O is a refinement since it	

Table 1: Summary of euthanasia studies in mice and rats with several agents and flow rates.



/ N ₂ O CO ₂ / N ₂		♂ ♀ C57BL/6J mice	GF 20% CO ₂ :60% N ₂ O	[20%] CO ₂ 96.76 ± 7.9		(pH, arterial partial pressure of oxygen, lactate); behaviour (rearing, jumping).	shortens the time to LOC by 10% without triggering any obvious increase in behavioural signs of aversion or distress.	
			GF 20% CO ₂ :60% N ₂	[27%] CO ₂ 112.46 ± 6.9				
CO	Makowska, 2009 ⁴⁷	♂ Wistar rats	GF 3% *	[5% ± 0.6%] / 104 ± 24	NA	Approach-avoidance (food rewards).	All animals exposed to CO exhibited convulsions after they were recumbent, but it is not clear if they were unconscious when this occurred.	
			GF 6% *	[5.5% ± 0.5%] / 64 ± 5				
			GF 7% **	[5.1% ± 0.6%] / 53 ± 7				
	Each flow rate was compared to Air delivered at a flow rate of: * 63%; ** 78%							
Ar			GF 40% - 120% *	NA	NA			

Table 1: Summary of euthanasia studies in mice and rats with several agents and flow rates.



	Makwoska, 2008 ⁴⁵	♂ Wistar rats	GF 120% – 239%**				Approach-avoidance (food rewards).	Ar is not a suitable alternative to CO ₂ for the euthanasia of rats.	Sound or air currents associated with gas entry were not the cause of aversion.
	Each flow rate was compared to Air delivered at a flow rate of: * 63%; ** 120-239%								
Halo Iso	Makwoska, 2009 ⁴⁰	♂ Wistar rats	Halo *	[2%]	158 ± 55	NA	Approach-avoidance (food rewards).	Halothane and isoflurane are aversive; however more 'humane' than CO ₂ exposure.	
				[2.5%]	138 ± 7				
				[3.25 %]	114 ± 3				
				[5%]	88 ± 16				
			Iso *	[1.25 %]	153 ± 14				
				[2%]	135 ± 14				
				[2.5%]	111 ± 8				

Table 1: Summary of euthanasia studies in mice and rats with several agents and flow rates.



				[3.75%]	79 ± 18				
	*in 63% O ₂								
Iso Sevo o	Bertolus, 2015 ⁴⁴	♂ Sprague Dawley rats	[8%] Sevo	* 89 (IQR = 81–91) ** 85.5 (IQR = 78–88)	NA	* Aversion-avoidance; ** Approach-avoidance.	GF Sevo and Iso are similarly aversive; they are a 'humane' alternative to CO ₂ exposure, if no recent exposure to those anaesthetics occurred.		
			[5%] Iso	* 80 (IQR = 78–80) ** 79 (IQR = 69–82)					

Ar: Argon; CO: Carbon Monoxide CO₂: Carbon dioxide; GF: Gradual-fill; Halo: Halothane; IQR: Inter-Quartile Range; Iso: Isoflurane; NA: Not applicable; LOC: Loss of consciousness; LOPWR: Loss of pedal withdrawal reflex; N₂: Nitrogen; N₂O: Nitrous Oxide; PF: Pre-fill; Sevo: Sevoflurane.